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INHIBITION OF MICROSOMAL METABOLISM AND CHEMICAL ONCOGENESIS IN CULTURE BY NAPHTHALENE QUINONES[1]

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A series of naphthalene diols, quinones, and related compounds were examined for their ability to inhibit mixed-function oxidase activities in liver microsomes obtained from rats which had been pretreated with 3-methylcholanthrene (3-MC) or phenobarbital (PB). Using benzo(a)pyrene monooxygenase as a measure of mixed-function oxidase activity, it was found that phenanthrene-9,10-quinone was the most active compound tested with a K_I = 0.79 μM. Phenanthrene-9,10-quinone did not affect cytochrome c reductase but did inhibit aminopyrine N-demethylase and p-nitroanisole-O-demethylase in both 3-MC and PB-induced microsomes with almost identical inhibition constants. 1,2-Naphthoquinone exerted similar effects as phenanthrene-9,10-quinone on cytochrome c reductase, aminopyrine N-demethylase and p-nitroanisole-O-demethylase. Both quinones stimulated NADPH oxidase activity but the extent of this stimulation did not explain their inhibition of microsomal oxidation. Kinetic studies using benzo(a)pyrene monooxygenase with phenanthrene-9,10-quinone and 1,2-naphthoquinone indicated that they were noncompetitive with benzo(a)pyrene and mixed noncompetitive with NADPH. Both of these quinones inhibited benzo(a)pyrene induced oncogenic transformation in C3H10T1/2CL8 cells in culture in a dose response manner, presumably by inhibition of the cellular microsomal enzymes which activate benzo(a)pyrene. Phenanthrene-9,10-quinone and 1,2-naphthoquinone seem to inhibit microsomal oxidative processes by interaction at the level of cytochrome P-450 possibly with a cytochrome P-450-substrate-oxygen complex.

Please send requests for reprints to Dr. Stephen Nesnow

[1]Presented in part at the 69th Annual Meeting of the American Association for Cancer Research, April, 1978 (Nesnow *et al.*, 1978).

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INTRODUCTION

Naphthalene quinones stimulate both rabbit (Sato *et al.*, 1962) and rat (Handa and Sato, 1976) microsomal NADPH and rat NADH oxidation (Tamura and Sato, 1977). These quinones also inhibit oxidative demethylation (Wills, 1972) cortisol metabolism (Kupfer and Peets, 1968), and anerobic glycolysis (Fukuoka *et al.*, 1957). It has been proposed that naphthalene quinones may inhibit these oxidative enzymes by the stimulation of NADPH oxidation, thus, rendering it unavailable for interaction with the microsomal electron transport system (Wills, 1972). This paper describes studies on the interactions of naphthalene quinones and related agents with various components of the microsomal electron transport pathway. A mechanism is proposed for the interaction of naphthalene quinones with mixed-function oxidases which does not use the stimulation of NADPH oxidation as its focal point. The effect of these quinones on the metabolism of benzo(a)pyrene and the oncogenic transformation of cells in culture by benzo(a)pyrene is also described.

MATERIALS AND METHODS

Chemicals and Radioactive Compounds

[G-^{3}H]-Benzo(a)pyrene (24-40 Ci/mmole) (Amersham/Searle, Arlington Heights, IL) was purified by high pressure liquid chromatography using a Dupont Zorbax-ODS column (4.6 mm ID × 24 cm) at 2000 psi (Dupont Instruments, Wilmington, DE) employing 100% methanol as the elution solvent (retention time, 6 min). Non-radioactive benzo(a)pyrene was added to the purified radioactive benzo(a)pyrene to adjust the specific activity to 12-25 μCi/μmol. Benzo(a)pyrene, NADP, NADPH, glucose-6-phosphate, bovine serum albumin, glucose-6-phosphate dehydrogenase, cytochrome c, vitamin K, menadione and 3-MC (Sigma Chemical Co., St. Louis, MO); 1,4-dihydroxynaphthalene (K and K Laboratories, Plainview, NY); and 2-acetyl-1-naphthol (Eastman Organic Chemicals, Rochester, NY) were used without further purification. 2-Phenylacetyl-1-naphthol was synthesized according to the procedure of Van Allen and Reynolds, (1969) and exhibited a mp of 96-97° (Bhumagara *et al.*, 1947). Spectrograde solvents, p-nitroanisole, p-nitrophenol, aminopyrine and all other inhibitors were purchased from Aldrich Chemicals, Milwaukee, WI, and phenobarbital-sodium from Mallinckrodt Chemical, St. Louis, MO.

Culture Media and Supplements

Eagle's Basal Medium with Earles' salts and L-glutamine, heat-inactivated fetal bovine serum, penicillin and streptomycin were purchased from Grand Island Biological Co., Grand Island, NY.

Preparation of Microsomes

Male Charles River CD rats (60-80 g) were maintained on lab chow and water *ad libitum*. Groups of at least 4 rats were induced either with PB, 80

mg/kg in 0.5 ml of saline injected ip on each of 3 successive days, or with 3-MC, 20 mg/kg in 0.5 ml of corn oil injected ip on each of 2 successive days. The rats were starved for 24 hr after the last treatment, and microsomes were prepared according to the method of van der Hoeven *et al.* (1974). Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as protein standard.

Enzyme Assays

Benzo(a)pyrene Monooxygenase Benzo(a)pyrene monooxygenase, an assay which measures the conversion of benzo(a)pyrene to benzo(a)pyrene diols, quinones and phenols, was assayed by the method of Nesnow *et al.* (1977). Microsomal protein concentration was 0.40 mg/ml using PB-induced microsomes and 0.20 mg/ml with 3-MC-induced microsomes. It has been determined that, in assays employing PB-induced rat liver microsomes, 91% of the total products are measured, while, with 3-MC-induced rat liver microsomes, 87% of the total products are measured. The concentration of inhibitor which causes a 50% reduction in enzymic activity (I_{50}) was determined for each inhibitor by constructing a dose-response relationship between log concentration of inhibitor and percent enzyme activity. At least four concentrations of inhibitor were used for each agent.

Cytochrome c Reductase Cytochrone c reductase was assayed according to Philips and Langdon (1962). In a 3 ml cuvette was placed 3 μmol of KCN, 150 nmol of cytochrome c, 0.150 mg of microsomal protein and 0.96 mmol of phosphate buffer pH 7.6. Inhibitor dissolved in acetone was then added and the reaction was begun upon addition of 0.126 μmol of NADPH. The reduction of cytochrome c was determined as the change in absorbance at 550 nm with time observed during 1-5 min of reaction. Samples containing the complete reaction mixture plus inhibitor, less NADPH, served as blanks. The nonenzymic reduction of cytochrome c was determined in samples containing the complete reaction mixture less microsomal protein.

NADPH Oxidase NADPH oxidation was determined by the decrease in absorption at 340 nm (Kupfer and Peets, 1968). A 3 ml incubation mixture contained 146 μmol of phosphate buffer pH 7.0 and 1.5 mg of PB induced microsomal protein. Inhibitor was added 1 min prior to initiation of the assay with 0.3 μmol of NADPH. The change in absorbance at 340 nm was monitored over 1-5 min of reaction and calculations employed an extinction coefficient of 6220 for NADPH. The rate of non-enzymic oxidation of NADPH due to the added inhibitors was subtracted from each point. Each assay was performed in triplicate with the coefficient of variation being 16%.

Aminopyrine N-Demethylase A 4 ml incubation mixture contained 200 μmol of Tris buffered to pH 7.5, 15 μmol of $MgCl_2$, 40 μmol of glucose-6-phosphate, 3.0 μmol of NADP, 4 units of glucose-6-phosphate dehydrogenase and 4 mg of microsomal protein (Yang and Strickhart, 1974). Inhibitor was added and 1 min later aminopyrine (20 μmol) was added to start the reaction. Samples were incubated with shaking at 37° for 15 min at which time the

reaction was stopped with 0.5 ml of 25% aqueous $ZnSO_4$ and 0.5 ml of saturated $Ba(OH)_2$. After mixing and centrifugation, a 1 ml aliquot of supernatant was incubated with 1 ml of Nash reagent and 1 ml of water. The optical density at 412 nm was read after incubating for 8 min at 58°. Formaldehyde enriched samples served as standards for quantitation and recovery. Each sample was adjusted for the absorbance due to the inhibitors.

p-Nitroanisole-O-Demethylase p-Nitroanisole-O-demethylase was assayed by the enzymatic conversion of p-nitroanisole to p-nitrophenol. A 2 ml incubation mixture contained 200 μmol of Tris buffered to pH 7.4, 15 μmol of $MgCl_2$, 20 μmol of glucose-6-phosphate dehydrogenase. Inhibitor was added 1 min prior to initiation of the assay with substrate, p-nitroanisole, 5 μmol. After incubation at 37° with shaking for 10 min the reaction was stopped with 3 ml of cold dichloromethane. After mixing a 2 ml aliquot of the organic layer was removed and extracted with 3 ml of 1 N NaOH. The absorbance of the NaOH layer was measured at 400 nm and quantitation based on an extinction coefficient of p-nitrophenolate of 18,200. Samples were adjusted for overall recovery (91%) and for the absorbance due to the inhibitors.

C3H10T1/2CL8 Oncogenic Transformation Assay

The mouse embryo cell line (C3H10T1/2CL8) used in these experiments was derived from a C3H mouse by Reznikoff *et al.* (1973a,b), has been previously described, and was kindly donated by Dr. Charles Heidelberger. Cells were incubated in humidified incubators with an atmosphere of 5% CO_2 in air at 37° and were grown in Eagle's Basal Medium supplemented with 10% heat-inactivated fetal bovine serum. Cultures were routinely checked for evidence of Mycoplasma contamination and were found to be Mycoplasma-free. In the assay, cells were plated onto dishes (1000/dish) in 5 ml of medium (12 dishes/experimental group), and 24 hr later treatment was begun. The treated dishes received quinone dissolved in acetone (0.5%). Three hrs later Benzo(a)pyrene (4 μM) was added to the cultures and incubated for 24 hrs. The media was then removed and the cells given fresh complete media supplemented with penicillin (100 units/ml) and streptomycin (50 μg/ml). Media was changed weekly until the cells reached confluency, whereupon the fetal calf serum concentration was reduced to 5%.

Control cultures received only acetone and were handled in the same manner as treated cells. Cytotoxicity tests were performed for each experimental group with the same protocol as used in the transformation assay, except that each dish was plated with 200 cells (6 dishes/experimental point) and that the dishes were stained 7 to 10 days after plating.

At 6 weeks the dishes were washed with 0.9% NaCl solution, fixed with methanol, stained with Giemsa, and then scored for oncogenic transformation. Three different morphological types of foci have been described, upon treatment of C3H1OT1/2CL8 cells with hydrocarbon oncogens. Only type II and type III foci have been scored, as these have been shown to produce sarcomas when injected into irradiated C3H mice (Reznikoff *et al.*, 1973b).

RESULTS

Inhibition of Benzo(a)pyrene Monooxygenase

A series of naphthalene derivatives were examined for their ability to inhibit the metabolism of benzo(a)pyrene (benzo(a)pyrene monooxygenase) using hepatic microsomes from rats pretreated with 3-MC or PB. It has been reported that 3-MC and PB induce different types of cytochrome P-450 hemoproteins with respect to their structure, molecular weight, substrate specificity, and product formation (Lu and West, 1978). The potency of these agents evaluated as I_{50}, the concentration of inhibitor which inhibits 50% of the enzymatic reaction, is listed in Table 1. The most potent inhibitors in this group

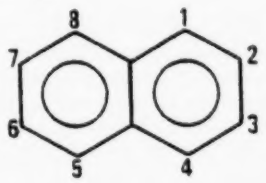
TABLE 1. Inhibition of Benzo(a)pyrene Monooxygenase[1] from Rat Liver Microsomes Induced with 3-Methylcholanthrene or Phenobarbital

Inhibitor[3]	I_{50}[2] 3-MC Induced Microsomes	PB Induced Microsomes
1-Naphthylmethanol	>1000	>1000
Hydroquinone	>1000	>1000
Phenol	>1000	500 ± 72
p-Cresol	>1000	310 ± 32
Naphthalene	>1000	195 ± 21
1,3-Dihydroxynaphthalene	>1000	172 ± 18
2-Phenylacetyl-1-naphthol	>2000	36 ± 8
2-Acetyl-1-naphthol	1000 ± 150	140 ± 18
2-Nitro-1-naphthol	800 ± 48	270 ± 30
1,7-Dihydroxynaphthalene	395 ± 20	33 ± 5
p-Phenylphenol	255 ± 14	103 ± 4
2-Naphthol	235 ± 8	32 ± 3
1-Naphthol	200 ± 5	22 ± 2
1,6-Dihydroxynaphthalene	185 ± 8	69 ± 8
Benzoquinone	130 ± 10	300 ± 21
2-Methyl-1-naphthol	130 ± 7	47 ± 5
1,5-Dihydroxynaphthalene	100 ± 6	50 ± 4
4-Amino-1-naphthol·HCL	13 ± 2	25 ± 3
1,4-Dihydroxynaphthalene	8.9 ± 1	14 ± 2
1,2-Diacetoxynaphthalene	3.4 ± 0.3	11 ± 2

[1]Benzo(a)pyrene Monooxygenase is a radiometric assay for the enzymatic oxidation of benzo(a)pyrene. This assay measures between 87-91% of all the benzo(a)pyrene metabolites formed.

[2]I_{50} is the concentration of inhibitor which inhibits 50% of the enzymatic reaction and is expressed in $\mu M \pm$ SD for four replicate samples: Benzo(a)pyrene concentration was 60 μM and the specific activity of untreated controls was 3-MC: 3.57 ± 0.15 and PB: 1.02 ± 0.12 nmoles/min/mg protein. Protein concentration in assay was: 3-MC: 0.200 mg/ml, PB: 0.400 mg/ml.

[3]The numbering system of naphthalene is:



were 1,4-dihydroxynaphthalene and 1,2-diacetoxynaphthalene. Dihydroxynaphthalenes had varying activity, from 3 to greater than 1000μM, depending on the ring location of each hydroxyl group. Conjugated diols (i.e., 1,2-; 1,4-; 1,5-dihydroxynaphthalenes) were more active than non-conugated ones (i.e. 1,3-; 1,6-; 1,7-dihydroxynaphthalenes). 1-Naphthols substituted in the 2 position had varying activity depending on the nature of the substituent (cf 2-acetyl-1-naphthol, 2-nitro-1-naphthol and 2-methyl-1-naphthol).

The naphthalene quinones studied for inhibitory activity (Table 2) group into two classes, 1,2-quinones and 1,4-quinones. The 1,4-quinone is the basic structural unit of the vitamin K series and within the group of 1,4-naphthoquinones tested there was a wide range of inhibitory activity. Substituents played a large role in determining the activity with the order in decreasing activity being 5-hydroxyl, 2-methylthio >H >2-methyl >>2,3-oxide >2-methyl-3-phytyl, 2-hydroxy. Within the series of 1,2-naphthoquinones, the order in decreasing activity was 3,4-benzo, H >>4-sulfonate. The activity of these agents is probably related to a combination of structural features and in their ability to accept electrons as seen by their standard oxidation-reduction potentials (Table 2). Phenanthrene-9,10-quinone, the most potent of the agents tested, has also been found to inhibit benzo(a)pyrene monooxygenase (unpublished data) in hepatic microsomes obtained from rats treated with Aroclor 1254, pregnenolone-16α-carbonitrile, 5,6-benzoflavone, or corn oil.

TABLE 2. Naphthalene Quinones as Inhibitors of Benzo(a)pyrene Monooxygenase

	I_{50}[1]		
Naphthalene Quinone	3-MC Induced Microsomes	PB Induced Microsomes	Redox Potential, $E_{O'}$[2]
Lawsone	>1000	1035 ± 150	−0.139
Vitamin K_1	>1000	1000 ± 125	−0.05
Menadione Oxide	>500	500 ± 47	
1,2-Naphthoquinone-4-sulfonate	31 ± 5	41 ± 4	
Menadione	18 ± 2	18 ± 3	
1,4-Naphthoquinone	9.4 ± 0.3	16 ± 2	+0.036
2-Methylthio-1,4-naphthoquinone	5.2 ± 0.7	3.6 ± 0.5	
5-Hydroxy-1,4-naphthoquinone	3.1 ± 0.2	9.2 ± 1.1	
1,2-Naphthoquinone	4.5 ± 0.8	9.2 ± 1.2	+0.143
Phenanthrene-9,10-quinone	3.2 ± 0.4	3.8 ± 0.6	

[1] I_{50} is the concentration of inhibitor which inhibits 50% of the enzymatic reaction and is expressed in μM ± SD or four replicate samples: Benzo(a)pyrene concentration was 60 μM and the specific activity of untreated controls was 3-MC; 3.57 ± 0.15 and PB: 1.02 ± 0.12 nmoles/min/mg protein. Protein concentration assay was 3-MC: 0.200 mg/ml, PB: 0.400 mg/ml.

[2] $E_{O'}$, in volts, is the standard oxidation potential at pH 7.0 (Rao and Hayon, 1973; Loach, 1976).

However, some of the naphthalene quinones exhibited different I_{50} values with respect to the two types of microsomal protein used. The I_{50} values of these agents (1,4-naphthoquinone; 5-hydroxy-1,4-naphthoquinone and 1,2-naphthoquinone) were found to be directly dependent on the amount of microsomal protein in the assay. With either PB or 3-MC induced microsomes (Fig. 1) the inhibitory action of 1,2-naphthoquinone was inversely proportional to the protein concentration. The inhibitory action of phenanthrene-9,10-quinone did not exhibit such protein dependency with either microsome type. The inhibitory effects of 7,8-benzoflavone, a potent inhibitor of 3-MC induced mixed-function oxidase activity (Weibel *et al.*, 1971), was also independent of protein concentration.

Inhibition of Other Mixed-Function Oxidase Activities

Both phenanthrene-9,10-quinone and 1,2-naphthoquinone inhibited the N-demethylation of aminopyrine and the O-demethylation of p-nitroanisole (Table 3). The inhibition constants for these microsomal enzymatic oxidative reactions were identical to those found with benzo(a)pyrene monooxygenase for phenanthrene-9,10-quinone and again were independent of the type and amount of microsomal protein. 1,2-Naphthoquinone also inhibited both the N- and O-demethylation reactions but the I_{50} values (19-23 μM) differred from those found in the benzo(a)pyrene monooxygenase assay (4-9 μM). The protein concentration used in the N- and O-demethylation assays was 1.00 mg/ml for both 3-MC and PB induced microsomes. An extrapolation of the data obtained for the variation of the I_{50} with microsomal protein concentration using the benzo(a)pyrene monooxygenase assay (Fig. 1) predicts an I_{50} at 1.00 mg/ml of 18-21 μM, a value which agrees well with the observed values for 1,2-naphthoquinone inhibition of N or O-demethylation. When the aminopyrine N-demethylase assay was conducted at 0.500 mg/ml protein (3-MC induced) or 0.250 mg/ml protein PB induced), the observed and predicted I_{50} values for 1,2-naphthoquinone were 11.5 ± 2.9; 11.8 μM and 8.9 ± 0.8; 7.0 μM, respectively.

Cytochrome c Reductase Studies

No inhibition of cytochrome c reduction was observed with either inhibitor for both types of microsomal protein (Table 3). 1,2-Naphthoquinone was found to stimulate both the enzymatic (170% of control at 200 μM) and non-enzymic reduction of cytochrome c. A concentration of 200 μM was sufficient to catalyze the non-enzymic reduction of cytochrome c at a rate of 327 nmoles cytochrome c reduced/min.

NADPH Oxidase Studies

Both 1,2-naphthoquinone and phenanthrene-9,10-quinone were evaluated for their effect on NADPH oxidation. Both quinones stimulated NADPH oxidase activity approximately threefold when incubated with PB induced microsomes (Fig. 2). This stimulation was detectable at 5 μM, a value roughly equal to the inhibitory activity (I_{50}) of phenanthrene-9,10-quinone in several

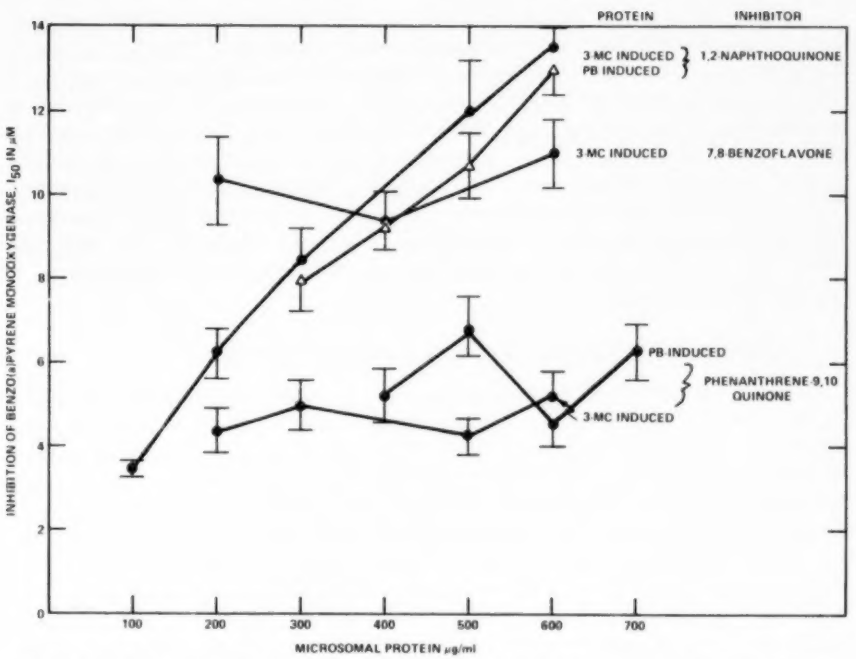


FIGURE 1. Inhibition of benzo(a)pyrene monooxygenase as a function of microsomal enzyme concentration. The inhibition caused by each agent was determined as an I_{50} by performing each assay at 4 doses/protein concentration/agent. In the absence of inhibitor, the reaction rate for PB and 3-MC induced microsomes was 1.02 and 3.57 nmoles benzo(a)pyrene oxidized/min/mg protein at 37°, respectively. Each point represents the mean of three determinations. The vertical lines represent SD.

oxidative microsomal reactions. Both quinones catalyzed the non-enzymic oxidation of NADPH with 1,2-naphthoquinone at 100 μM capable of an oxidation rate of 30 nmole NADPH oxidized/min. The greatest oxidation rate observed was with microsomes and phenanthrene-9,10-quinone, a rate of 90 nmoles NADPH oxidized/min.

Kinetics of the Inhibition of Benzo(a)pyrene Monooxygenase

A kinetic analysis of the inhibition of benzo(a)pyrene monooxygenase by phenanthrene-9,10-quinone and 1,2-naphthoquinone was undertaken to elucidate the interaction between these quinones and cytochrome P-450. Each quinone was assayed in the presence of varying amounts of either substrate, benzo(a)pyrene or NADPH, using 3-MC induced microsomes at 0.300 mg/ml protein concentration. Dixon plots of the inhibition data were drawn and the kinetic constants obtained. Both quinones exhibited uncompetitive kinetics with benzo(a)pyrene and pure mixed non-competitive kinetics (Wong, 1975)

TABLE 3. Inhibition of Microsomal Enzymes by Naphthalene Quinones

Naphthalene Quinone	I_{50}[1] Cytochrome c Reductase[2]		Aminopyrine N-Demethylase[3]		p-Nitroanisole O-Demethylase[4]	
	3-MC	PB	3-MC	PB	3-MC	PB
1,2-Naphthoquinone	> 200[5]	>200	22.4±6.2	22.6±1.2	19.1±1.9	19.1±1.5
Phenanthrene-9,10-quinone	>200	>200	4.8±0.3	3.3±0.4	3.3±0.4	4.5±0.5

[1] I_{50} is the concentration of inhibitor which inhibits 50% of the enzymatic reaction and is expressed in μM ± SD for four replicate samples.

[2] The specific activity of untreated controls was 3-MC: 194 ± 15 and PB: 480 ± 21 nmoles cytochrome c reduced/min/mg protein. Each assay received 0.050 mg/ml of microsomal protein. Substrate concentration was 50 μM.

[3] The specific activity of untreated controls was 3-MC: 4.84 ± 0.40 and PB: 11.78 ± 0.73 nmoles formaldehyde formed/min/mg protein. Each assay received 1.00 mg/ml of microsomal protein. Substrate concentration was 5000 μM.

[4] The specific activity of untreated controls was 3-MC: 2.38 ± 0.06 and PB: 2.69 ± 0.03 nmoles p-nitrophenol formed/min/mg protein. Each assay received 1.00 mg/ml of microsomal protein. Substrate concentration was 2500 μM.

[5] 1,2-Naphthoquinone stimulates the enzymatic reduction of cytochrome c. This agent also catalyzes the non-enzymic reduction of cytochrome c.

with NADPH. The K_m observed for benzo(a)pyrene (14.6 ± 0.5 μM) agrees with a value of 22 μM previously reported in the literature (DePierre *et al.*, 1975) while that for NADPH was 108 ± 2.1 μM. The K_i observed for 1,2-naptho-quinone was 2.8 ± 0.09 μM and for phenanthrene-9,10-quinone was 0.79 ± 0.11 μM.

Inhibition of Benzo(a)pyrene Mediated Oncogenesis in Cultured Cells

C3H10T1/2CL8 mouse embryo fibroblasts were pretreated with either 1,2-naphthoquinone or phenanthrene-9,10-quinone and then treated with benzo(a)pyrene for 24 hr. Neither 1,2-naphthoquinone nor phenanthrene-9,10-quinone were themselves cytotoxic to C3H10T1/2CL8 cells from 0.03 to 3 μM. Benzo(a)pyrene (4 μM) was cytotoxic to these cells (60-83% survival). With the combined treatment of 1,2-naphthoquinone and benzo(a)pyrene or phenanthrene-9,10-quinone and benzo(a)pyrene, the cytotoxicity due to the carcinogen was unchanged (Fig. 3). With increasing concentrations of 1,2-naphthoquinone the transformation frequency was decreased by 63% at maximum inhibition. Phenanthrene-9,10-quinone also inhibits benzo(a)pyrene mediated transformation in dose response manner in C3H10T1/2CL8 cells with a maximal inhibition of approximately 90% at 0.3 μM (1/10 the benzo(a)pyrene concentration).

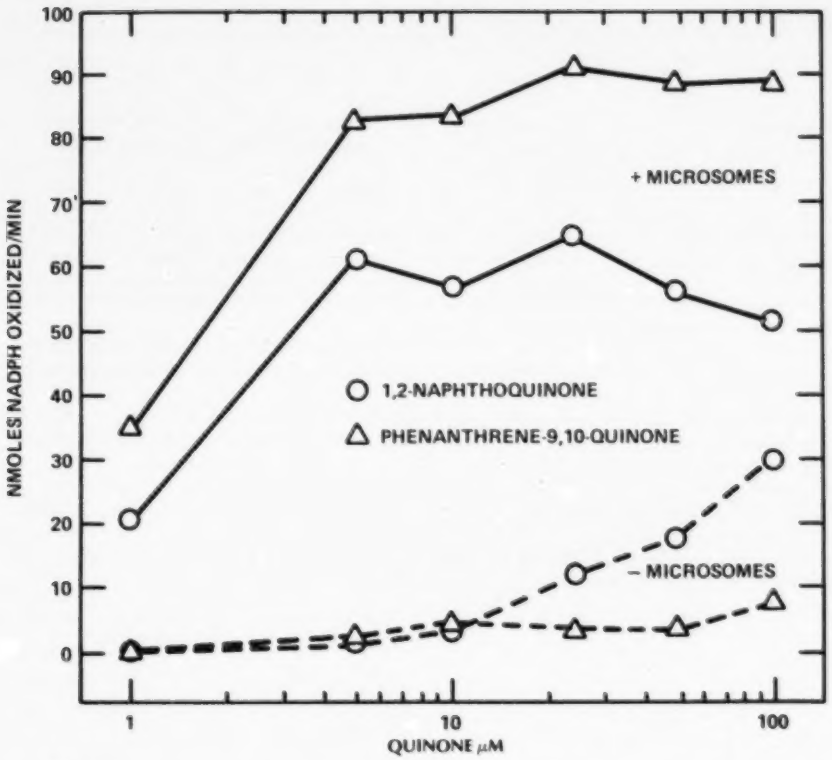


FIGURE 2. The stimulation of microsomal NADPH oxidation and non-enzymic NADPH oxidation by quinones. The rate of non-enzymic oxidation of NADPH due to the added inhibitors was subtracted from each point which is the mean of triplicate determinations.

DISCUSSION

In the course of these studies, it was observed that the inhibitory action of certain naphthalene quinones 1,4-naphthoquinone, 5-hydroxy-1,4-naphthoquinone and 1,2-naphthoquinone, was directly proportional to the microsomal protein concentration in the assay. 1,2-Naphthoquinone reacts with eye lens proteins to form covalent adducts and 1,2-naphthoquinone and 1,4-naphthoquinone react with cysteine, probably at their reactive double bonds (van Heynigen and Pirie, 1967; Nishibayashi *et al.*, 1967). The binding of naphthalene quinones to microsomal protein would, therefore, cause the effective inhibitory concentration to be dependent on protein concentration. Differences between those quinones whose inhibitory activity was inversely proportional to the microsomal protein concentration (Table 2) and those which were not, may be due to the nature of the substituents at the reactive double bond either enhancing or inhibiting covalent binding.

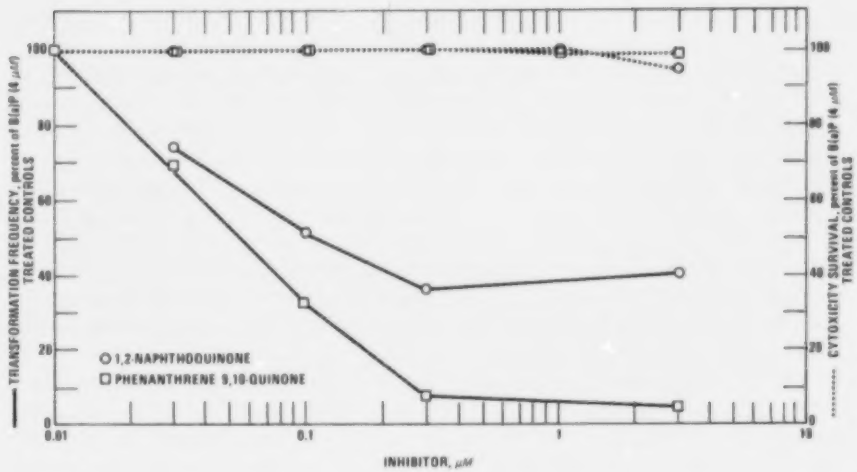


FIGURE 3. The effects of naphthalene quinones on benzo(a)pyrene mediated cytotoxicity and transformation of C3H10T1/2CL8 cells. Cells were pretreated with the quinones for 3 hrs at which time they were treated with 4 μM benzo(a)pyrene for 24 hrs. No spontaneous transformants were observed in the control dishes. The plating efficiency in the control dishes averaged 19% while that in the benzo(a)pyrene treated dishes averaged 14%. Benzo(a)pyrene at 4 μM transformed 1.3% of the cells at risk.

Of all the naphthalene quinones tested as inhibitors of microsomal oxidative processess, the most potent was phenanthrene-9,10-quinone, an inhibitor which is nonspecific towards the type of cytochromes (induced or non-induced) with which it interacts. These results are in contrast to other known inhibitors of mixed-function oxidase activity such as SKF 525A and 7,8-benzoflavone which are specific inhibitors of PB-induced and 3-MC-induced microsomes, respectively (Weibel *et al.*, 1971; Nesnow *et al.*, 1977). Because of this lack of specificity, studies were performed on the effects of naphthalene quinones on NADPH oxidase, cytochrome c reduction, and cytochrome b_5 reduction, other components of the microsomal electron transport pathway.

Both 1,2-naphthoquinone and phenanthrene-9,10-quinone stimulated the enzymic oxidation of NADPH 2.5-3 fold at concentrations which inhibited 50% of the mixed-function oxidase activity. These quinones also non-enzymatically catalyzed the oxidation of NADPH to NADP but only at high concentrations. Wills (1972) observed similar stimulations of enzymic NADPH oxidation with the related quinones, menadione and vitamin K_5. It has been suggested that naphthalene quinones stimulate enzymic NADPH oxidation by functioning as electron carriers between NADPH cytochrome c reductase and molecular oxygen (Handa and Sato, 1976; Nishibayashi *et al.*, 1967). The stimulation of NADPH oxidation by quinones cannot, however, explain the inhibition of benzo(a)pyrene oxidation because the NADPH generating system used in the benzo(a)pyrene assay maintains a constant NADPH level at or near 1mM.

The reduction of cytochrome c was not inhibited by either 1,2-naphthoquinone or phenanthrene-9,10-quinone at concentrations up to 200 μM (Table 3). The apparent dichotomy between naphthalene quinone stimulation of NADPH oxidation and lack of inhibition of cytochrome c reduction is explained by comparing the quantities of NADPH initially present in the reductase assay (126 nmole) with those consumed (16-20 nmole) due to naphthalene quinone stimulation of NADPH oxidation. Although some electrons are being diverted from reaching the flavoprotein by the quinones, there is more than enough NADPH left to reduce the cytochrome c. The lack of inhibition of cytochrome c reduction by naphthalene quinones is not clear evidence that they do not inhibit the transfer of electrons from the reductase to cytochrome P-450. It is possible for the reductase to reduce the quinone which as demonstrated will reduce cytochrome c.

Within the electron transport chain of the endoplasmic reticulum, electrons can flow from NADPH cytochrome c reductase to cytochrome b_5 (Estabrook *et al.*, 1975). p-Cresol has been found to stimulate the aerobic reoxidation of cytochrome b_5 reduced by NADPH in rat liver microsomes with a concomitant increase in O_2 consumption. This phenol stimulation was also found to be inhibited by cyanide (Oshino and Sato, 1971). p-Cresol had virtually no effect on the oxidation of benzo(a)pyrene by induced rat liver microsomes (Table 1) and potassium cyanide (1mM) did not reverse the inhibitory effects of 1,2-naphthoquinone or 1,4-naphthoquinone with 3-MC induced microsomes (data not shown). These two pieces of evidence indicate that naphthalene quinones are not acting at the levels of cytochrome b_5.

Both phenanthrene-9,10-quinone and 1,2-naphthoquinone were found to inhibit the oxidation of benzo(a)pyrene and the demethylation of aminopyring and p-nitroanisole. The inhibition constants for phenanthrene-9,10-quinone were the same regardless of the cytochrome type, substrate, or substrate concentration. The same was true for 1,2-naphthoquinone after adjusting for protein dependency. This indicated that these quinones were interacting with the microsomal electron transport chain at a rate limiting step or species central to these oxidative processes. Matsubura *et al.* (1976), Guengerich *et al.* (1976), and Miwa *et al.* (1978) have suggested the rate limiting step to be the transfer of a second electron to the cytochrome P-450-substrate-oxygen complex.

Kinetic analysis of the inhibition of enzymatic oxidation revealed that the naphthalene quinones were uncompetitive with benzo(a)pyrene, indicating that these inhibitors could be acting on a cytochrome P-450-substrate-oxygen complex. NADPH is essentially a source of electrons and provides the electrons necessary for benzo(a)pyrene oxidation via cytochrome P-450. The observation of pure, mixed non-competitive kinetics with NADPH in this multisubstrate, multicomplex reaction cannot be explained mechanistically although may be the result of quinone accepting electrons from both a cytochrome P-450-substrate-oxygen complex and from NADPH directly.

The results indicate that phenanthrene-9,10-quinone and 1,2-naphthoquinone seem to interact at several sites of the microsomal electron transport complex (Fig. 4). Electrons can be diverted directly from NADPH or from a

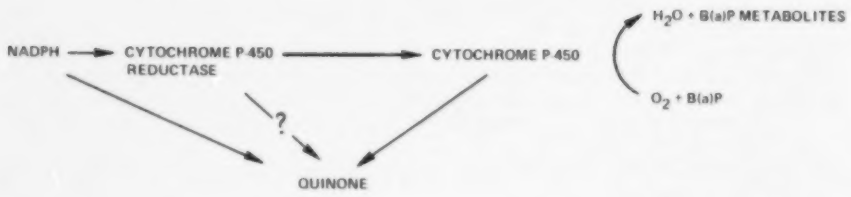


FIGURE 4. Interactions of naphthalene quinones with the electron transport complex within the endoplasmic reticulum.

cytochrome P-450 complex to quinone. However, the major effect of these quinones on the inhibition of microsomal oxidative processes seems to be at the site of cytochrome P-450. While the data presented does not identify any one particular cytochrome P-450-complex, it does suggest that this interaction is not at the step of cytochrome P-450 binding to substrate.

It has been shown that 7,8-benzoflavone can reduce carcinogen-induced oncogenic transformation of cells in culture by inhibiting the enzymes which metabolize carcinogens to their activated forms. (Nesnow and Heidelberger, 1976). Since naphthalene quinones can inhibit carcinogen metabolism, it was, therefore, of interest to examine the potential of these compounds to effect chemical oncogenesis of cells in culture. In the present studies, naphthalene quinones inhibited the oncogenic effects of benzo(a)pyrene in C3H10T1/2CL8 cells in culture (I_{50}~0.05-0.10 μM). The results presented here suggest that quinone inhibition of oncogenic transformation of C3H10T1/2CL8 cells occurred via inhibition of the cellular microsomal enzymes which activated benzo(a)pyrene.

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